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RECONVERSION OF DETERGENT- AND SULFHYDRYL REAGENT-PRODUCED P-420 TO P-450 BY POLYOLS AND GLUTATHIONE

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SUMMARY

After treatment with detergents or sulfhydryl reagents, the cytochrome pigment P-420 can be converted back to P-450 by treatment with polyols or reduced glutathione. This reconversion of P-420 to P-450 is highly dependent on temperature, pH, concentration of sodium cholate and incubation time, but ionic strength was of little influence. However, after treatment with alcohols or ketones, P-420 could no longer be reconverted to P-450 by polyols and reduced glutathione.

The presence of 20 % (v/v) glycerol stabilizes P-450 for more than 1 week.

INTRODUCTION

In 1958, KLINGENBERG[1] and GARFINKEL[2] found a microsomal CO-binding pigment which was later demonstrated to be a *b*-type cytochrome and named P-450 by OMURA AND SATO[3].

This cytochrome has abnormal spectrophotometric properties in the presence of CO and can be converted into P-420 by protease, urea, sulfhydryl reagents or detergents[4,5]. P-420 has been shown to possess the typical absorption spectrum of other known hemoproteins[3].

The present paper reports studies on the mechanism of the reversibility of the conversion of P-420 to P-450 for the elucidation of the unusual properties of P-450.

MATERIALS AND METHODS

Microsomal preparations

The microsomes used were prepared from fresh rabbit liver in this laboratory by a modification of the method of MITOMA *et al.*[6]. This microsomal preparation was found to be practically free of hemoglobin on spectrophotometric analysis in the presence of CO and was shown to contain less than 1 % contamination with mitochondria on a protein basis by measurement of succinic oxidase activity.

Analytical procedures

P-450 and P-420 contents were determined by the method of OMURA AND SATO[3].

Abbreviations: ESR, electron spin resonance; PCMB, *p*-chloromercuribenzoic acid.

CO was purchased from Nippon Sanso Co. The purity of the gas was shown by gas chromatography to be 99.7 %.

PCMB concentration was determined by the method of BOYER[7].

The protein content was determined by the biuret method of GORNALL AND BARDAWILL[8] after the addition of 1 % sodium cholate to the sample to remove turbidity. Crystalline bovine serum albumin was used as a standard. The additional absorption in the biuret reaction due to heme in the test samples was avoided by using $CuSO_4$-free biuret reagent.

Chemicals

Urea was obtained from Wako Pure Chemicals Co. It was recrystallized three times from 70 % ethanol before use. The other reagents used were obtained commercially.

Spectrophotometric measurements

Difference spectra of microsomal preparations were measured in a Cary model 14 spectrophotometer at room temperature using cuvettes of 1 cm optical path.

Measurements of ESR spectra

ESR spectroscopy was performed with a Varian V-4500-10A spectrometer with a 100-kcycles/sec field modulation unit and spectra were generally obtained at a sample temperature of 100° K. Quartz sample tubes of 3 mm internal diameter were used on the Varian variable-temperature attachment. The field modulation amplitude used was 15 Gauss unless otherwise stated.

Measurements of the reversibility of the conversion of P-420 to P-450

Detergents and sulfhydryl reagents were incubated aerobically with microsomal suspensions at 15° for 10 and 30 min, respectively, unless otherwise indicated. After incubation, the test solutions were diluted 2-fold with 50 % (v/v) polyols, GSH or other solutions. Then spectrophotometric measurements were made after storage at 15° for 5 min.

The initial rapid conversion of P-450 to P-420 with detergents, or sulfhydryl reagents occurred instantaneously and the steady state was little affected by the experimental conditions over a 60-min period. However, after longer periods, the P-420 which coexisted with P-450 at the steady state in these experiments, was unstable under aerobic conditions and disappeared.

Ionic strength

The ionic strengths of the KH_2PO_4–Na_2HPO_4 buffer used were estimated from the table of BOYD[9].

RESULTS

It has been found that P-450 which is closely associated with microsomal Fe_x (refs. 10, 12) can be readily converted to P-420 with proteases, phospholipases, monohydric alcohols, amides, ketones, ureas, phenols, anilines, guanidine salts, nitriles, sulfhydryl reagents, dioxane, detergents and under conditions such as at lower and

higher pH values[4,5,10,11]. The so-called P-420 magnetically is of at least two types; one type has an ESR signal of microsomal Fe_x which contains high-spin[13] and low-spin states, and another type gives the decomposed ESR signal of microsomal Fe_x. The P-420 which has the ESR signal of microsomal Fe_x was found spectrophotometrically to be converted to P-450 by polyols, GSH or dialysis.

Conversion of P-420 formed with sodium cholate to P-450 with polyols

Fig. 1a shows that the P-450 disappearing with increase in the concentration of sodium cholate was inversely related to P-420 appearance. However, as shown in Fig. 1b, the content of microsomal Fe_x remained nearly unchanged, as reported in the presence of deoxycholate[10]. After these test solutions had been incubated at

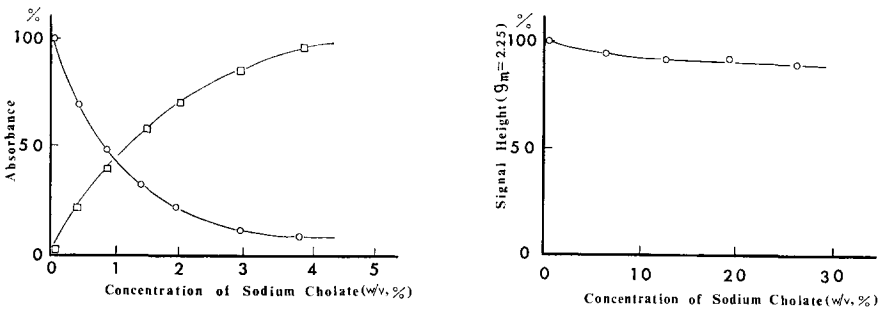


Fig. 1. a. Relationship between microsomal CO-binding pigments at various concentrations of sodium cholate. The test samples were incubated at 15° for 10 min with various concentrations of sodium cholate. The ordinate shows the percentage of the absorbance between 450 mμ and 490 mμ for P-450, and the absorbance between 420 mμ and 490 mμ for P-420. Microsomal protein, 2 mg/ml; 0.1 M phosphate (pH 7.0). ○—○, P-450; □—□, P-420. b. Effect of sodium cholate on microsomal Fe_X. The test samples were incubated at 15° for 10 min with various concentrations of sodium cholate. The height of the ESR signals of microsomal Fe_x was measured at $g_m = 2.25$. The points show the percentages of the height of the initial ESR signal. Microsomal protein, 20 mg/ml; 0.1 M phosphate (pH 7.0).

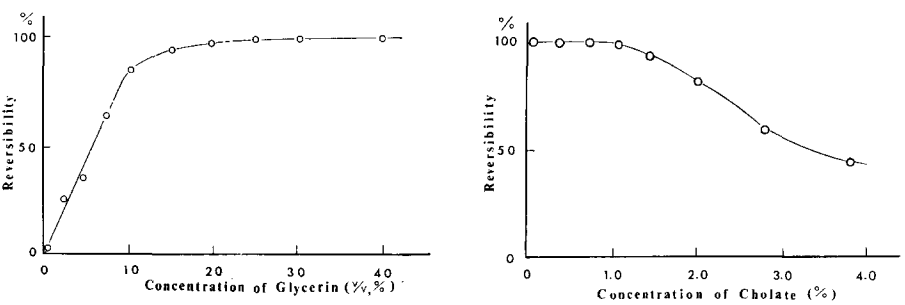


Fig. 2. Reversibility of the conversion of P-420 to P-450 at various concentrations of glycerol. 2% (w/v) sodium cholate was added to microsomal suspensions in 0.1 M phosphate at pH 7.0. Measurements were made as described before. The reversibility is shown as a percentage of that in 25% glycerol. Microsomal protein, 2 mg/ml.

Fig. 3. Reversibility of P-420 to P-450 at various concentrations of sodium cholate with glycerol. Various concentrations of sodium cholate were added to microsomal suspensions in 0.1 M phosphate at pH 7.0 and 25% (v/v) glycerol was added to the test solutions after incubation as described before. The control contained only buffer and sodium cholate at the final concentrations indicated. Microsomal protein, 2 mg/ml.

pH 7.0, as previously described, there was little conversion of P-420 to P-450 on dilution of the solution although this was possible by treatment with polyols such as glycerol and glycol.

Fig. 2 shows the reversibility of P-420 to P-450 with various concentrations of glycerol. When the concentration of the glycerol was less than 10 % (v/v), the conversion of P-420 to P-450 was incomplete. The same result was also obtained in the presence of substances such as ethylene glycol and propylene glycol.

Table I shows the reversing activity of various reagents in the conversion of P-420 to P-450. This reversibility was not observed on addition of aminopyrine, barbiturates, monohydric alcohols, dioxane, amides or ketones.

Fig. 3 shows the reversibility of P-420 to P-450 at various concentrations of sodium cholate in the presence of a final concentration of 25 % (v/v) glycerol.

TABLE I

REVERSIBILITY OF THE CONVERSION OF P-420 FORMED WITH SODIUM CHOLATE TO P-450 WITH VARIOUS REAGENTS

Polyols and related compounds were added to the test solutions at the final concentrations shown in the table. The other conditions were as described before. The results were shown as percentages of the reversibility of P-420 to P-450 in the presence of 25 % (v/v) glycerol. Microsomal protein, 2 mg/ml; 0.1 M phosphate (pH 7.0).

Reagent	*Concentration*	*Reversibility* (%)
Polyols		
Ethylene glycol	25 % (v/v)	100
Polyethylene glycol	25 % (v/v)	100
Propylene glycol	25 % (v/v)	82
Ethylene glycol monobutyl ether	25 % (v/v)	0
Diethylene glycol monoethyl ether	25 % (v/v)	100
Glycerol	25 % (v/v)	100
Erythritol	25 % (w/v)	47
Ribitol	25 % (w/v)	32
Sorbitol	25 % (w/v)	30
Sucrose	25 % (w/v)	25
Alcohols		
Methyl alcohol	25 % (v/v)	0
Ethyl alcohol	25 % (v/v)	0
tert.-Amyl alcohol	10 % (v/v)	0
Ketones		
Acetone	25 % (v/v)	0
Acetonylacetone	25 % (v/v)	0
Miscellaneous		
Dimethylsulfoxide	25 % (v/v)	55
Dimethylformamide	25 % (w/v)	0
N,*N*-Dimethylacetamide	25 % (w/v)	0
Dioxane	25 % (v/v)	0
Sodium propionate	25 % (w/v)	0
GSH	0.05 M	0
Sodium phenobarbital	400 mg/ml	0
Aminopyrine	30 mg/ml	0
(Dialysis)	(15 h at 5°)	30

Fig. 4 shows the effect of ionic strength on the conversion of P-420 to P-450 by glycerol. The changes in ionic strength had little effect on the reversibility.

Fig. 5 shows the effect of incubation time on the reversibility of P-420 to P-450 in the presence of 2% sodium cholate. Thus, full reversibility was observed for up to about 15 min on incubation at 15°. The initial phase of irreversibility appears after between about 20 and 30 min incubation. This result is not due to lability of P-420 and P-450 under aerobic conditions. The graph of the effect of incubation time on the irreversibility of P-420 to P-450 shows a clearly biphasic curve.

Fig. 6 shows the effect of incubation temperature on the reversibility of the conversion of P-420 to P-450 at pH 7.0. This reversibility is highly temperature dependent and complete reversibility is observed below 18°. On the other hand, on warming at 35° for 10 min, P-420 was scarcely converted to P-450 even on subsequent

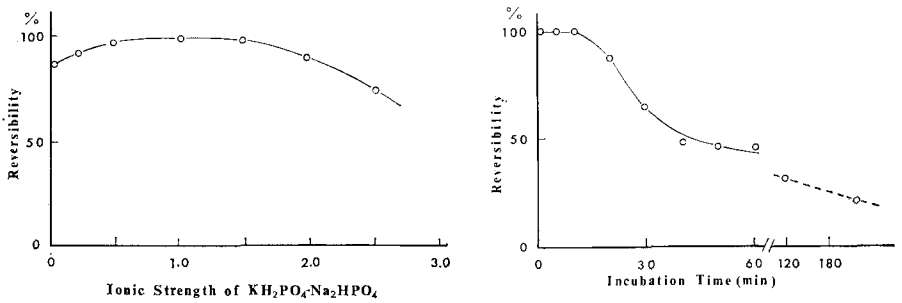


Fig. 4. Effect of ionic strength on the reversibility of P-420 to P-450. 2% (w/v) sodium cholate was added to microsomal suspensions of various ionic strengths in KH_2PO_4–Na_2HPO_4 buffer at pH 7.0 and 15°. Incubation was carried out as described before and then 25% glycerol was added to the solutions. Microsomal protein, 2 mg/ml.

Fig. 5. Effect of incubation time on reversibility of 2% (w/v) sodium cholate-produced P-420 to P-450 by glycerol. The abscissa shows the incubation time before the addition of 25% (v/v) glycerol. Microsomal protein, 2 mg/ml; 0.1 M phosphate (pH 7.0).

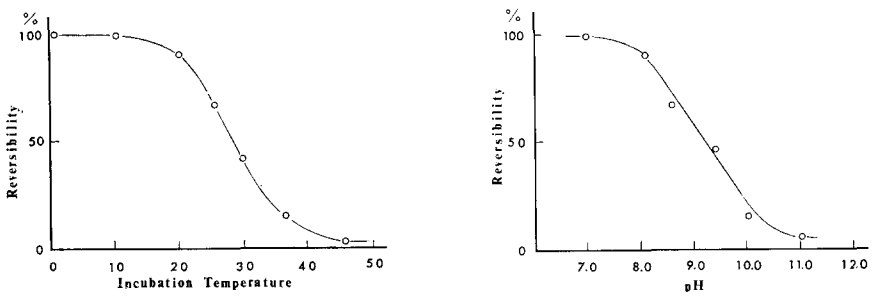


Fig. 6. Effect of incubation temperature on reversibility of P-420 to P-450. The abscissa shows the temperature for the 10-min incubation period. The microsomal suspension in 0.1 M phosphate at pH 7.0 was incubated with 2% (w/v) sodium cholate before the addition of 25% (v/v) glycerol. Microsomal protein, 2 mg/ml.

Fig. 7. pH dependence of reversibility of P-420 to P-450 with glycerol. Liver microsomes (protein, 2 mg/ml) were suspended in 0.1 M phosphate buffer at below pH 8.0 or glycine–NaOH buffer at above pH 8.5. 25% (v/v) final concentration of glycerol was added to the microsomal suspensions. The other conditions are as described in the text.

rapid cooling to 5° in the presence of 25 % glycerol. The P-450 of untreated microsomes was not converted to P-420 over the range of temperatures examined.

Fig. 7 shows the effect of pH on the reversibility of P-420 to P-450 with glycerol. This figure shows that between pH 7.0 and 8.0, reversibility was high. As reported previously, microsomal Fe_x decreases with increase in the pH (ref. 10). Accordingly, the above facts suggest that P-420 which does not have the ESR signal of microsomal Fe_x is scarcely converted to P-450 with glycerol.

Fig. 8 shows the stabilization of P-450 by glycerol. When P-450 was incubated at 16° and pH 7.0, the spontaneous decomposition of P-450 gave a first-order curve under aerobic conditions. The presence of glycerol prevented the decomposition of P-450, and P-450 was stable for more than 5 months in the presence of 75 % glycerol.

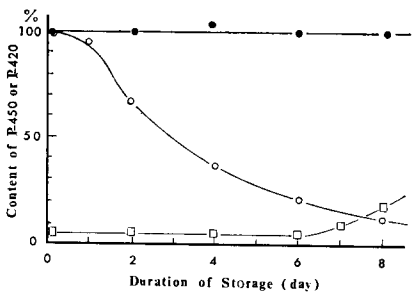


Fig. 8. Stabilization of P-450 by 20% (v/v) glycerol during aerobic storage at 16°. Microsomal protein, 2 mg/ml; 0.1 M phosphate (pH 7.0). ●—●, P-450 in the presence of glycerol; ○—○, P-450 in the absence of glycerol; □—□, P-420 in the absence of glycerol.

Reversibility of the conversion of P-420 obtained with PCMB to P-450 by GSH and glycerol

It is known that P-450 is converted to P-420 in the presence of sulfhydryl reagents[4]. When P-450 was treated with PCMB, as described previously, the disappearance of P-450 corresponded to the increase in concentration of P-420 with

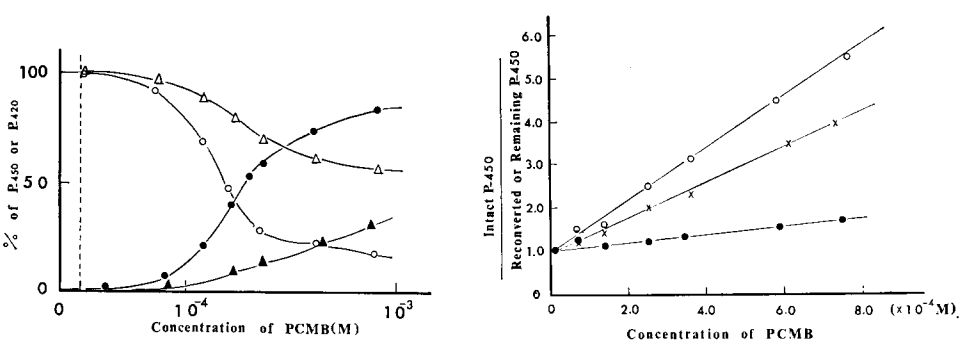


Fig. 9. Left: Change of P-450 to P-420 with various concentrations of PCMB and reversibility of P-420 to P-450 by GSH. Microsomal protein, 2 mg/ml; 0.1 M phosphate (pH 7.0); GSH, 0.02 M; temperature, 15°. ○—○, P-450 *plus* PCMB; ●—●, P-420 after addition of PCMB to P-450; △—△, P-450 *plus* PCMB *plus* GSH; ▲—▲, PCMB-produced P-420 *plus* GSH. Right: Ratio of intact P-450 to reconverted or remaining P-450 by treatment with and without GSH or glycerol in the presence of various concentrations of PCMB. ○—○, P-450 *plus* PCMB; ●—●, P-450 *plus* PCMB *plus* GSH; ×—×, P-450 *plus* PCMB *plus* glycerol.

increasing concentration of PCMB (Fig. 9, left). This result was similar to that with sodium cholate. After treatment with PCMB, P-420 could be converted to P-450 by addition of GSH, cysteine or glycerol. P-420 obtained by treatment of other sulfhydryl reagents, mercaptide-forming reagents, such as $HgCl_2$, $CuSO_4$ and $CdCl_2$, also facilitated the conversion to P-450 with GSH or glycerol. On the other hand, after treatment with alkylating agent such as CH_3I, there was only slight conversion of P-420 to P-450 on treatment with GSH or glycerol.

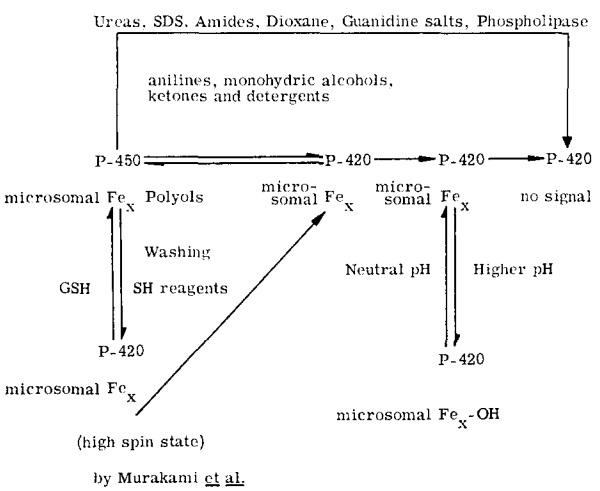
Fig. 9 (right) shows that PCMB-produced P-420 can be converted to P-450, partially by the addition of glycerol and almost completely by GSH. The reversibility is shown by the ratio of the P-450 of untreated microsomes to remaining P-450 after PCMB treatment or to reconverted P-450 by the addition of glycerol or GSH.

DISCUSSION

On combination with CO it is known that other hemoproteins have a Soret band below 430 mμ. However, on combination with CO, P-450 has a Soret band at 450 mμ. It is still not known what structure in CO–P-450 gives this unusual property.

P-450 is converted to P-420 by detergents or sulfhydryl reagents *etc.* After treatment with detergents or sulfhydryl reagents, this P-420 can be reconverted to P-450 by addition of polyols and GSH. This may be because polyols and GSH uncouple the detergents and sulfhydryl reagents from P-420 so that its conversion to P-450 again becomes possible or because some linkages in P-420 which are attacked on treatment with detergents and sulfhydryl reagents can again be formed on treatment with polyols and GSH under appropriate conditions.

After treatment with ureas, amides, monohydric alcohols, ketones, nitriles, guanidinium salts or sodium dodecyl sulfate, P-420 thus obtained could not be converted to P-450 by polyols and GSH. However, after treatment with monohydric



Scheme I. Scheme of possible interconversions of P-450 and P-420. The arrows indicate the direction of the conversion between P-450 and P-420.

alcohols or ketones, P-420 formed could be partially converted to P-450 by washing with 0.1 M phosphate at pH 7.0. The above results are summarized in Scheme I.

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